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Radiolabeled Exosomes for the Early Detection of Metastases and to Predict Breast Cancer Premetastatic Niche

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14. ABSTRACT **ABSTRACT** Since current diagnostic tools fail to detect breast cancer (BC) metastatic spread at a very early stage, we proposed a new approach for the early detection of metastatic disease in BC patients using cancer cell derived particles known as exosomes as a guide. We hypothesized that exosomes tagged with appropriate radionuclides could be used as molecular imaging probes to study and non-invasively image metastatic spread, and to detect pre-metastatic niches using single-photon emission computed tomography (SPECT) or Positron emission tomography (PET). During this report period we have: 1) isolated and characterized exosomes from a series of BC cell lines of different metastatic capacity and tropism, 2) radiolabeled isolated BC exosomes with different radionuclides, such as ^{111}In, ^{64}Cu, ^{89}Zr. We plan to 3) study the uptake and subsequent intracellular transport of radiolabeled exosomes by different cell types involved in pre-metastatic niche formation, 4) to study the in vivo stability, pharmacokinetics and biodistribution of radiolabeled exosomes following intravenous administration in naive and pathological mouse models. 5) Study the biodistribution and tumor uptake of radiolabeled exosomes (derived from peripheral blood of BC patients) in BC.

15. SUBJECT TERMS
breast cancer, exosomes, pre-metastatic niche, metastasis

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DOD Grant/Contract (Award Number W81XWH-13-1-0249)

YEAR 2 OF RESEARCH REPORT

Grant Title: Radiolabeled exosomes for the early detection of metastases and to predict breast cancer pre-metastatic niches

INTRODUCTION:

This grant is focused on the early detection of metastatic disease in patients with breast cancer (BC). This project takes advantage of the breakthrough knowledge in tumor-derived exosome tropism and exploits recent advances in the development of radioisotope based molecular imaging probes. We believe that non-imaging techniques, either single-photon emission computed tomography (SPECT) or positron emission tomography (PET) may offer an advantage to detect exosome distribution in metastatic organs and thus detect pre-metastatic niches *in vivo*. Our goal is to provide for the first time a framework for the prediction of metastatic spread and the diagnosis of pre-metastatic niche formation in BC based on preclinical studies using radiolabeled exosomes.

Summary of the tasks/aims proposed and achievements:

Specific Aim 1. To isolate and characterize fluorescently labeled exosomes from a series of BC cell lines of different metastatic capacity and tropism (timeframe, e.g., months 2-18). Dr. Lyden's group was and continues to be responsible for this task.
Specific Aim 1.1 To purify exosomes derived from MCF-7 and MDA-231 (available in 3 different clones and characterize, size, number and protein concentration of exosomes (timeframe, months 2-10).
Specific Aim 1.2 To prepare fluorescently labeled exosomes and determine the biodistribution in mice. 4 different exosome preparations will be studied in 40 mice (timeframe, months 2-10).

We have characterized the biodistribution of exosomes derived from breast cancer models. Our data suggest that exosomes from different cancer models recapitulate the metastatic organotropism of their cell of origin, therefore tumor exosomes could be potentially used to track organ specific metastasis.

We tested whether exosomes derived from MDA-MB-231 sub-clones that colonize lung, bone or brain (4175-LuT, 1833-BoT and 831-BrT, respectively) would also exhibit organ tropism. Exosomes from MDA-MB-231 sub-lines were phenotypically similar by NanoSight and electron microscopy (data not shown). Exosome biodistribution of the MDA-MB-231 variants varied 24-hours post-injection: lung-tropic 4175-LuT exosomes preferentially localized to lung with a>four-fold increase in exosome-positive cells compared to 1833-BoT ($P<0.001$) and 831-BrT ($P<0.001$) exosomes; (Fig. 1), whereas brain-tropic 831-BrT exosomes efficiently localized to brain with a >four-fold increase

compared to 1833-BoT (P<0.01) and 4175-LuT (P<0.01) exosomes (Fig. 1). Liver, and bone showed no significant differences in lung-, brain- or bone-tropic MDA-MB-231-derived exosome distribution (Fig. 1).

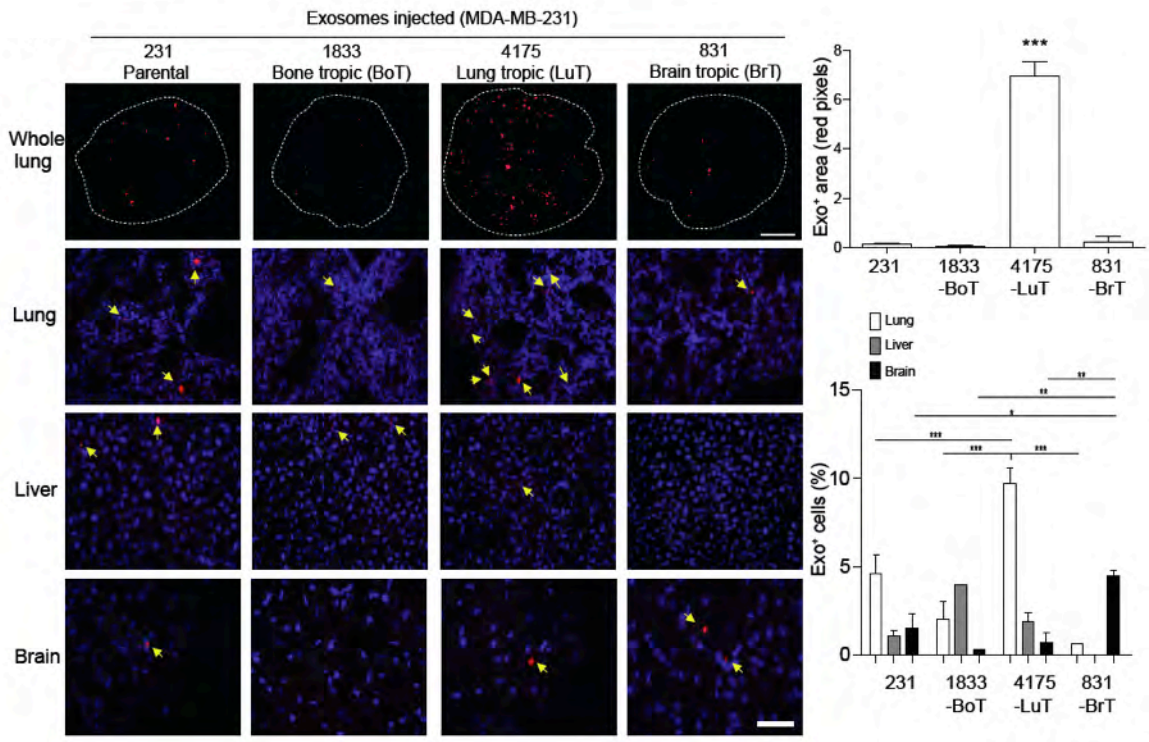

Figure 1. **Cancer cell-derived exosomes retain metastatic organotropism.** MDA-MB-231-derived exosome biodistribution. Upper panels represent Odyssey imaging (left, scale bar represents 500 μm) and quantification of exosome distribution (right, n = 3; bars depict average ± s.e.m. ***P <0.001 by one-way ANOVA). Upper-center, lower-center and lower panels represent fluorescence microscopy (left, arrows indicate exosome foci, scale bar represents 50 μm) and quantification of exosome distribution (right, n = 3-4 animals per experiment (3 independent experiments); error bars, ± s.e.m. *P <0.05; **P <0.01; ***P <0.001 by one-way ANOVA).

Specific Aim 2: To develop procedures and protocols to radiolabel exosomes derived from BC cell lines, MCF-7 and MDA-231 with different radionuclides in order to achieve radiolabeled exosomes of high specific activity, stability and specificity (timeframe, months 4-24).

Specific Aim 2.1. To radiolabel isolated BC exosomes with different radionuclides, such as ^{111}In, ^{64}Cu, ^{89}Zr

Specific Aim 2.2. To optimize the radiolabeling protocol in order to achieve a radioactive probe of high specific activity, stability and specificity.

Dr. Vallabhajosula's group is responsible for Specific Aim 2. During year 2, further progress has been made for this task testing additional radiolabeling methods for BC exosomes: A) radioiodination using I-124 and Iodogen method, B) radioiodination using I-131 and Bolton-Hunter method, C) NOTA conjugation and labeling with Ga-68 and D) DFO conjugation and labeling with Zr-89.
We aim to test a few more radiolabeling methods in year 3 and complete the proposed experimental part of the project with extended *in vitro* and *in vivo* testing for the final evaluation of the best method to radiolabel exosome preparations, in terms of preservation of exosome function post-labelling and sensitivity of exosome detection *in vivo*.

Results:

A) Radioiodination using Iodine-124 and the Iodogen Method

Experimental part:
During year 1, exosomes isolated from MDA-231 cells (lung-tropic) have been successfully radiolabeled with I-131 using the Iodogen method.

In year 2, the same Iodogen method has been used to label MDA-231 exosomes with I-124. I-124 is a positron-emitting radioisotope of iodine of great interest since it can used *in vivo* for PET imaging. A similar reaction protocol has been employed as in the case of I-131 with minor differences. Briefly:

In a PIERCE® pre-coated iodination tube and in a final volume of 0.20-0.30mL, exosomes were mixed with $Na^{124}I$ (0.3-1 mCi) in 1M PBS pH 7.4. $Na^{124}I$ was supplied by the cyclotron of Memorial Sloan Kettering Cancer Center. The reaction mixture was incubated at 33-34^{o}C for 1 h while gently swirled periodically on a shaker. The reaction was quenched by addition of an excess (50uL; 10mg/mL) of p-hydroxyphenylpropionic acid that serves as free I-124 scavenger. Unbound I-124 was removed by size exclusion chromatography on PD-10 columns pre-conditioned with 0.1M PBS pH 7.4, 0.25% BSA, a buffer that serves also as the elution buffer (0.5mL fractions collected). Labeling efficiency and RCP were determined by instant-thin layer chromatography (ITLC) on silica gel strips (Biodex or Agilent Technologies) and 85% MeOH or PBS as mobile phase. Moreover, a "mock" (radio)labeling was also performed following exactly the same protocol but using "cold" sodium iodide (NaI) instead. The purpose of this mock labeling was to check the effect of the radiolabeling procedure on exosome integrity using electron microscopy.

Results:
I-124 labeling yield reached 60-65%. Following purification with size exclusion chromatography RCP was >95% as shown by ITLC (**Figure 2**).

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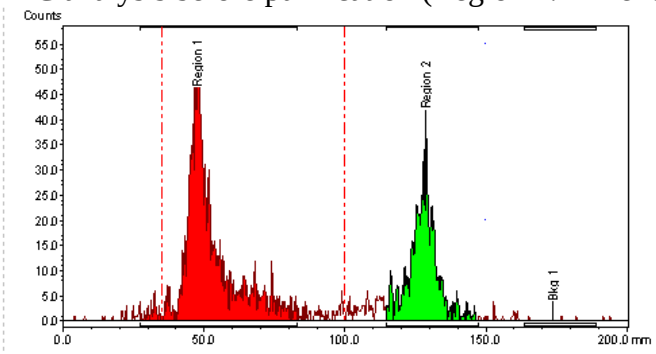
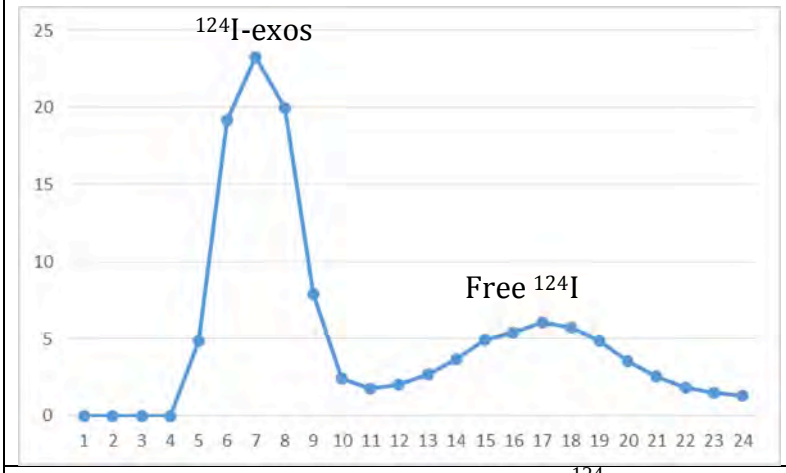
Figure 2

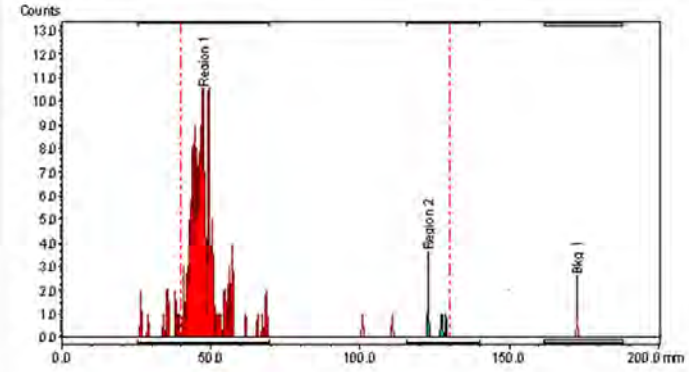
ITLC analysis before purification (Region 1: ^{124}I-exos and Region 2: free I-124):



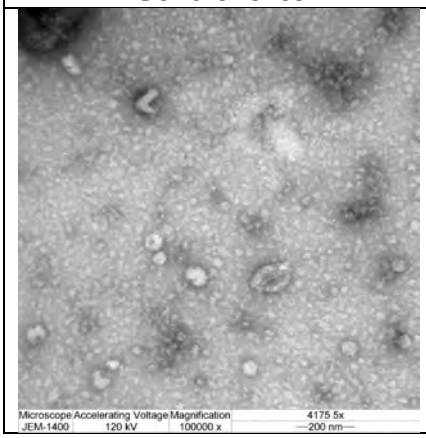
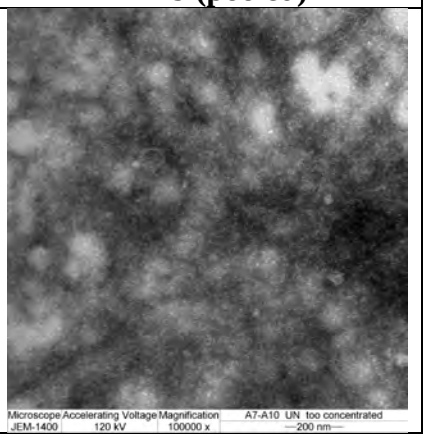
Size exclusion chromatography (purification):



ITLC analysis post purification (Region 1: ^{124}I-exos and Region 2: free I-124):



To determine how the iodination protocol affects the structural integrity of exosomes, the PD-10 fractions F7-F9 of the mock labeling were analyzed by electron microscopy together with control (untreated) exosomes. EM images are shown in **Figure 3**.

Figure 3	
Control exos	**F7-F9 (pooled)**
	

According to EM data, no exosome-like membrane vesicles are present in F7-F9 preparations suggesting that the Iodogen method is harsh for the exosomes and damages their integrity.

C) Radioiodination using Iodine-131 and the Bolton-Hunter method

The use of a Bolton-Hunter (BH) reagent is an alternative way of radioiodination of proteins that is performed under milder conditions compared to the Iodogen method. The Bolton-Hunter reagent is used to add tyrosine-like groups to end-terminal α-amino groups or ε-amino groups of lysine that result in increased protein iodination.

Experimental part:

1) Preparation of ^{131}I-BH reagent.

$Na^{131}I$ (in 0.1M NaOH from Perkin Elmer; ≥1 mCi) was mixed with equal volume of 1.0M Acetic acid and the mixture was dried by azeotropic distillation and acetonitrile.
5 mg of the BH precursor were dissolved in 400 microliters of acetonitrile. The mixture was allowed to stand for 10 min and then added to $Na^{131}I$ solution followed by solvent evaporation with a stream of dry argon at 90°C. After the solvent was removed completely, 125 uL of CH_3CN was added (with shaking or stirring) to dissolve the salts. Toluene (125 uL) was added and the solution was heated at 90°C for 30 min. Silica TLC (50% ethyl acetate, 50% hexanes) was performed to determine the labeling efficiency. The reaction mixture was purified by passing it through a silica sep-pak (treated with 2 x 2 mL hexanes) and the final product was eluted in 3mL ethyl acetate. Ethyl acetate was then removed by evaporation and the ^{131}I-BH reagent was dissolved in DMSO and stored at -20^{o}C till further use.

2) Labeling of MDA-231 exosomes with the ^{131}I-BH reagent.

70 ug of freshly isolated exosomes from MDA-231 cells (lung-tropic) were added to a ^{131}I-BH solution diluted in PBS in order to achieve 5% DMSO concentration (not toxic to

exos). The exosome labeling mixture was incubated at room temperature under mild stirring for 3h. A separate identical mixture was incubated for 24h.

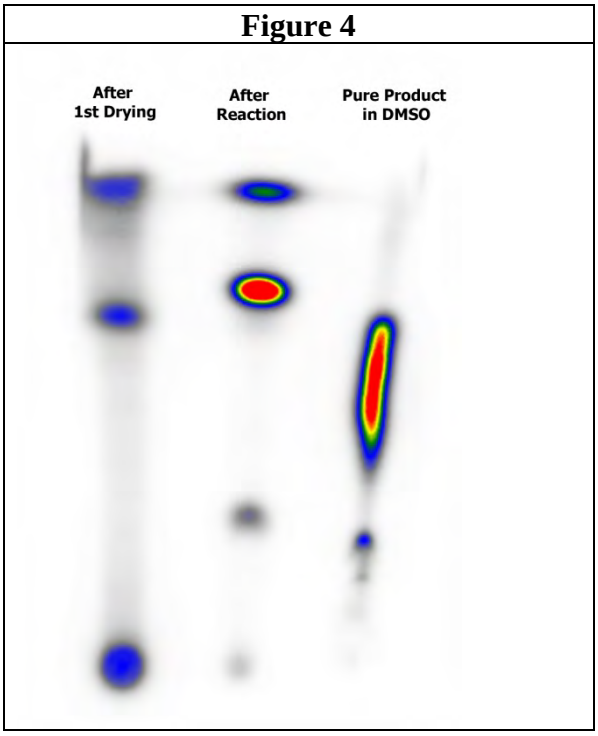
After completion of incubation, free ^{131}I-BH was removed by size exclusion chromatography on PD-10 columns pre-conditioned with 0.1M PBS pH 7.4, 0.25% BSA buffer. Labeling efficiency and RCP were determined by instant-thin layer chromatography (ITLC) on silica gel strips (Biodex or Agilent Technologies) and PBS as mobile phase.

Results:

1) Preparation of ^{131}I-BH reagent.

The overall yield of ^{131}I-BH reagent synthesis was 80%. ITLC analysis results are shown in **Figure 4**. The purity of the final ^{131}I-BH product was 94% (third lane on the right in the Radio ITLC).

Figure 4

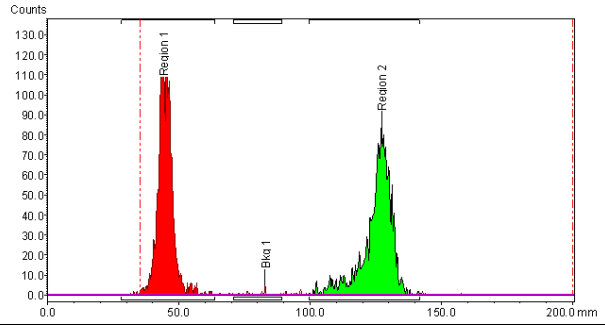


2) Labeling of MDA-231 exosomes with the ^{131}I-BH reagent.

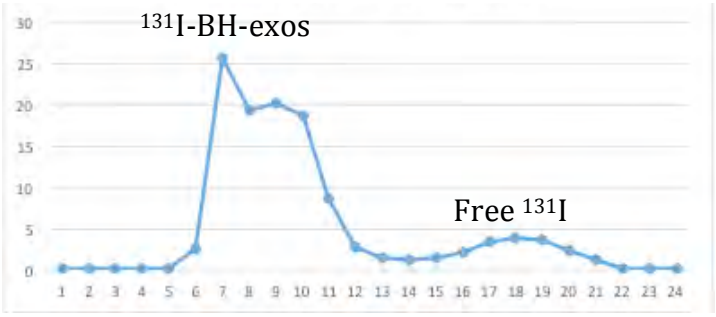
Both 3h and 24h reaction mixtures showed similar results. Labeling yield reached 45-50%. Following purification with size exclusion chromatography RCP was >95% as shown by ITLC. In **Figure 5** the representative results for the 3h incubation mixture are summarized. The 3h incubation labeling protocol was repeated 3x leading to same results. Mock labeling reactions were also performed in order to test the effect of the radiolabeling procedure on exosome integrity.

Figure 5

ITLC analysis before purification (Region 1: ^{131}I-BH-exos and Region 2: free I-131)

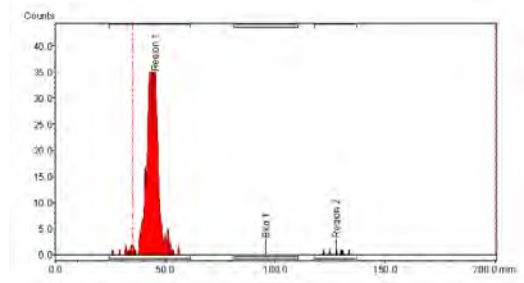


Size exclusion chromatography (purification):

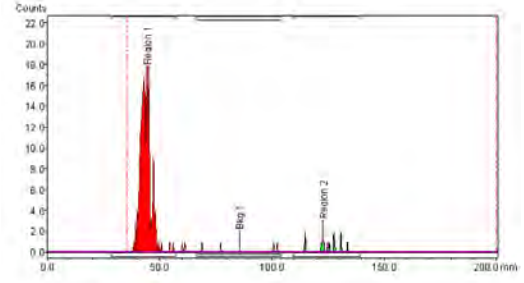


ITLC analysis post purification (Region 1: ^{131}I-BH-exos and Region 2: free I-131):

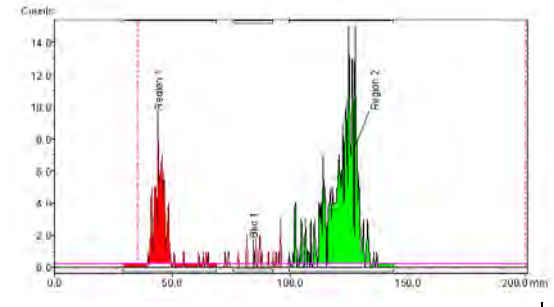
F7 (RCP: 98%)



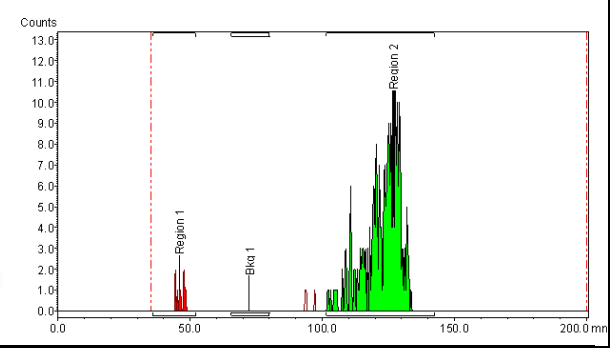
F8 (RCP: 96%)



F9 (RCP: 18%)



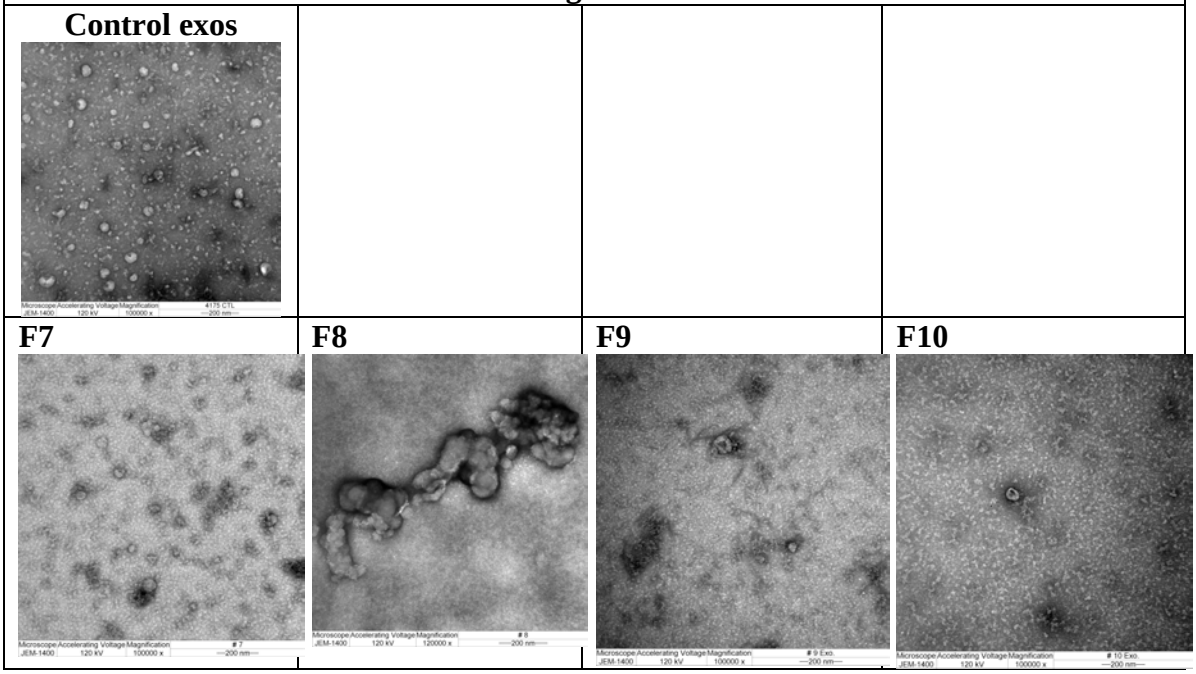
F10 (RCP: 3%)



As shown in Figure 5, fractions F7 and F8 contain highly pure radioiodinated exosomes (RCP>96%).

Representative EM images of the PD-10 fractions F7-F10 of the mock labeling are shown in **Figure 6**. According to EM data the Bolton-Hunter of radioiodination is well tolerated by exosomes that prereserve integrity following the reaction protocol.

Figure 6



D) Labeling with Gallium-68 (Ga-68)

Experimental part:

1) Conjugation of MDA-231 exosomes with NOTA chelator.

50uL of 20mg/mL NOTA-SCN in DMSO were mixed with 70 ug freshly isolated exosomes followed by dilution with PBS to a final volume of 1 mL (final DMSO concentration: 5%). The mixture was left to react overnight at ambient temperature. After 12h incubation, the conjugation solution was purified on a PD-10 column using PBS as eluate. Fractions F7-F10 were collected and then used for Electron Microscopy and labeling with Ga-68.

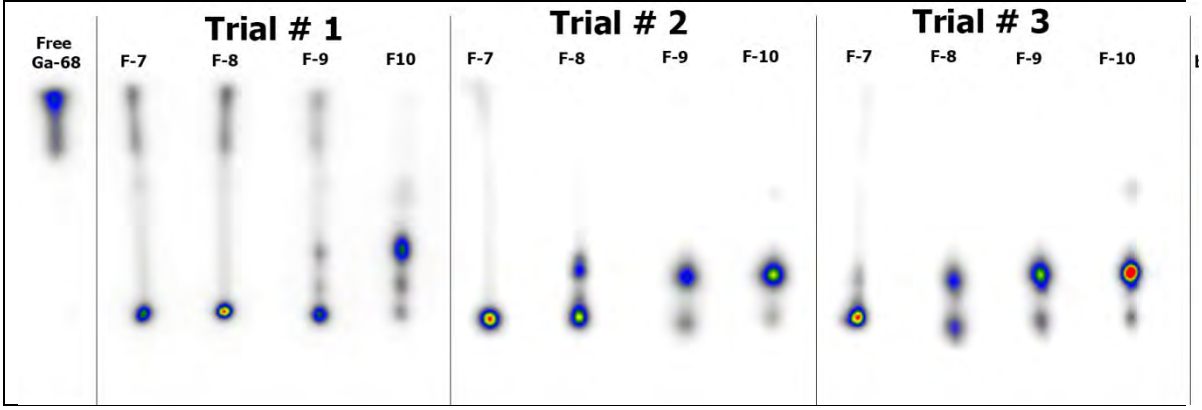
2) Labeling with Ga-68.

10 µl of 3N sodium acetate was first added to the vial containing 1 ml PBS with exosomes. Then, 200 ul of $^{68}GaCl_3$ eluate (370-390 MBq/ml) were added (final pH=5.5). The reaction mixtures were then incubated at ambient temperature for 15 min. Labeling yield was determined by ITLC analysis on Silica gel 60 plates and 0.1 M Citrate buffer (pH=4)/25% MeOH as mobile phase. Plates were developed using a Packard phosphorimager (Perkin Elmer, USA).

Results:

Three independent conjugation/labeling trials have been performed. The overall labeling yield of NOTA-modified exosomes with Ga-68 was 75% (**Figure 7).** Fractions F7-F10 were further analyzed by Electron microscopy (**Figure 8**). As shown below, EM data suggest that functionalization of exosomes with NOTA is a well-tolerated procedure.

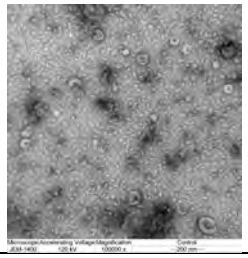
Figure 7



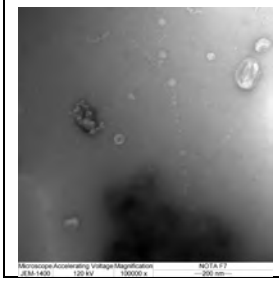
Fractions F7-F10 were further analyzed by Electron microscopy (**Figure 8**). As shown below, EM data suggest that functionalization of exosomes with NOTA is a well-tolerated procedure.

Figure 8

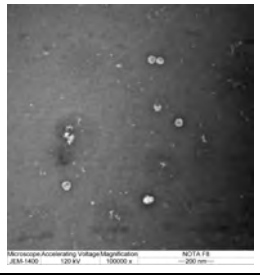
Control exos



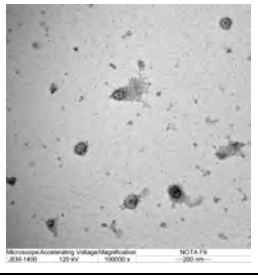
F7



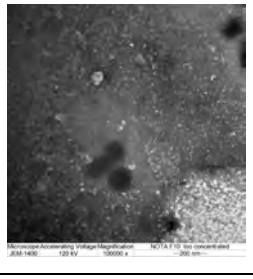
F8



F9



F10



D) Labeling with Zirconium-89 (Zr-89)

Experimental part:

1) Conjugation of MDA-231 exosomes with DFO (desferrioxamine) chelator.

50uL of 20mg/mL DFO-SCN in DMSO were mixed with 70 ug freshly isolated exosomes followed by dilution with PBS to a final volume of 1 mL (final DMSO concentration: 5%). The mixture was left to react overnight at ambient temperature. After 12h incubation, the conjugation solution was purified on a PD-10 column using PBS as eluate. Fractions F7-F10 were collected and then used for Electron Microscopy and labeling with Zr-89.

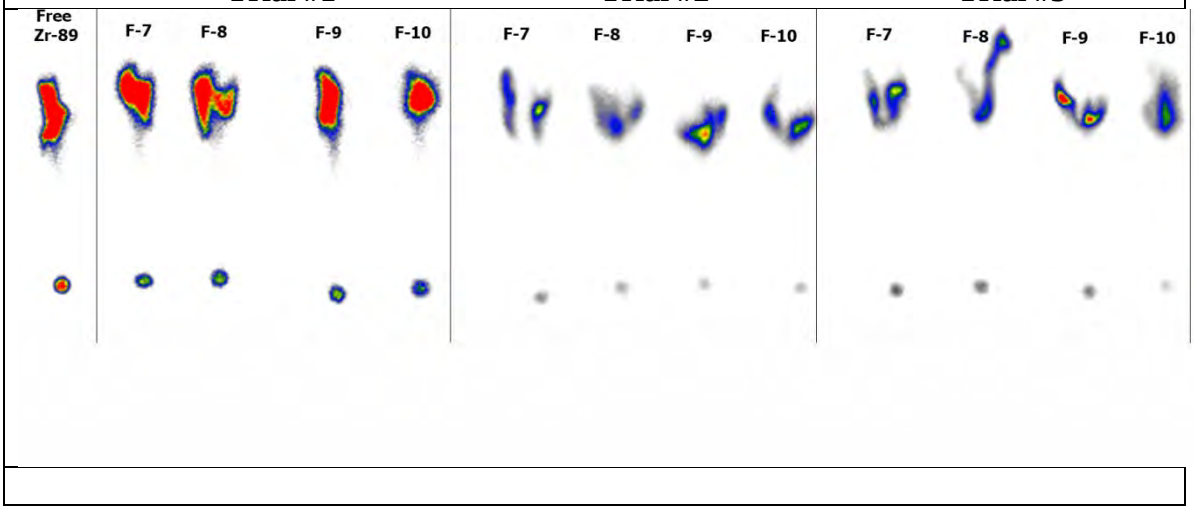
2) Labeling with Zr-89.

40 µl of 3N sodium acetate was first added to the vial containing 1 ml PBS with exosomes. Then, 10 ul of [^{89}Zr]Oxalate were added (Final pH=6.0). The reaction mixtures were then incubated at ambient temperature for 15 minutes. ITLC analysis was perfomed with Silica gel 60 plates and 0.1 M Citrate buffer (pH=4)/25% MeOH as mobile phase. Plates were developed using a Packard phosphorimager (Perkin Elmer, USA)

Results:

Three independent conjugation/labeling trials have been performed. The overall labeling yield of DFO-modified exosomes with Ga-68 was very poor for all trials and all fractions tested (**Figure 9).** It is concluded that the exosomes are unable to be functionalized with the DFO chelator at the conditions tested most probably due to the low solubility of DFO-SCN. Our effort to keep the DMSO concentration at 5% in order to protect the exosome integrity seemed to have a detrimental effect on DFO solubility that further affected conjugation to exosomes. Due to the poor Zr-89 labeling, EM analysis of DFO-modified exosome fractions F7-F10 was not performed.

Figure 9



Specific Aim 3. To study the *in vitro* uptake and subsequent intracellular transport of radiolabeled exosomes by different cell types, known to participate in the pre-metastatic niche formation mechanism, such as the primary tumor cells, BM cells, fibroblasts (NIH/3T3, lung) and endothelial cells (breast, lung, HUVEC). The binding,

internalization and intracellular trapping of radiolabeled exosomes and fluorescence labeled exosomes will be compared (timeframe, months 8-24).

Specific Aim 3.1 Determine the kinetics of labeled exosomal binding (and internalization) to tumor cells, BM cells, fibroblasts and endothelial cells.
Specific Aim 3.2 Evaluate the transference of exosomes from BC to stromal cells in vitro
Specific Aim 3.3 Determine the specific populations of bone marrow cells that can specifically take up exosomes from BC cell lines using flow cytometry.

We have continued the analysis to determine the kinetics and specific uptake of exosomes by bone marrow-derived, fibroblast and endothelial cells. Our *in vitro* data demonstrated that tumor-secreted exosomes can be uptaken by different stromal cells. To identify resident stromal cells taking up tumor exosomes in each organ, we intravenously injected red fluorescently-labeled exosomes from 4175-LuT, BxPC3-liver tropic or 831-BrT cells (Fig. 10). We determined that, 24 hours post-injection, both 1833-BoT and 4175-LuT exosomes promoted vascular leakiness prior to exosome uptake by specific cells in the lung (data not shown). These observations fit with our previous studies in melanoma exosomes, suggesting that exosomes permeabilize vessels allowing for exosome diffusion before uptake by parenchymal cells. Unexpectedly, we observed that the specific cell-type responsible for exosome uptake varied depending on the metastatic organ. 4175-LuT exosomes mainly co-localized with S100A4-positive fibroblasts and surfactant protein C-positive epithelial cells (40% and 30% of exosome-positive cells, respectively) in the lung (Fig. 10). In contrast, pancreatic cancer exosomes derived from liver-tropic BxPC3 cells fused with Kupffer cells (90% of exosome-positive cells; Fig. 10). Finally, analysis of the 831-BrT model revealed that exosomes interacted mainly with CD31-positive brain endothelial cells (Fig. 10). Collectively, these data demonstrate that specific tissue-resident stromal cells differentially uptake tumor exosomes in specific metastatic target organs.

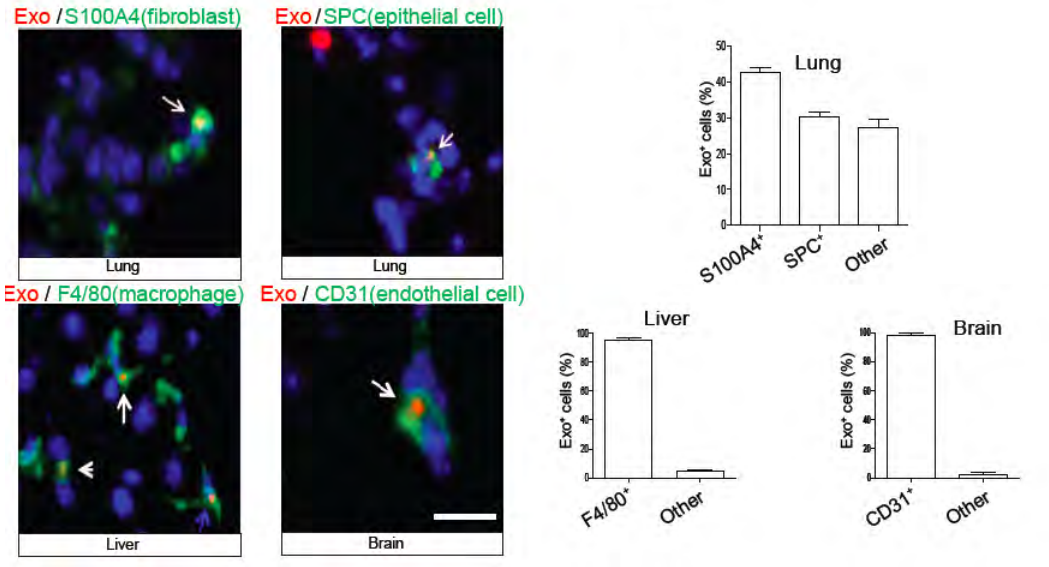


Figure 10. Resident cells specifically uptake tumor exosomes. Analysis of exosome (red) co-staining with different cell types. Upper panel, representative immunofluorescence microscopy image of lung tissue showing 4175-LuT exosome co-staining with S100A4 fibroblast marker and surfactant protein C (SPC) epithelial cell marker (green). Upper right graph, quantification by flow cytometry of the frequency of organotropic exosome-positive fibroblasts and epithelial cells. Lower left panel, liver tissue co-stained for macrophage marker F4/80 (green) and BxPC3 exosomes (red). Lower left graph, flow cytometric quantification of the frequency of organotropic exosome-positive macrophages. Lower right panel, brain tissue co-stained for endothelial cell marker CD31 (green) and 831-BrT exosome. Lower right graph, quantification of the frequency of 831-BrT exosome positive endothelial cells by immunofluorescence microscopy. Scale bar represents 30 μm. $n = 5$; error bars, ± s.e.m.

Specific Aim 4. To study the *in vivo* stability, pharmacokinetics, and biodistribution of radiolabeled exosomes following intravenous injection in naive and breast cancer high malignant (MDA-231) and low malignant (MCF-7) human breast cancer mouse models (timeframe, months 13-36).

Research staff from Dr. Vallabhajosula and Dr. Lyden will be working together on this task.

The design of in vivo studies in animal models depends very much on the success of in vitro stability studies and in vitro tumor cell binding studies with radiolabeled exosomes. A tentative plan is presented here to assess biodistribution.

Specific Aim 4.1 Identify 5 different radiolabeled exosomal preparations with optimal radiochemical purity, specificity and specific activity.

Specific Aim 4.2 Perform *in vivo* biodistribution studies at different time points (10 min, 1, 2, 4, 8, 24 hours) following administration of 0.1-0.3 mCi of radiolabeled (111In, 64Cu or 89Zr) exosomes based on μSPECT/PET/CT imaging system

Specific Aim 4.3 Based on imaging studies identify 2 time points for tissue distribution studies. Following sacrifice of mice (5 mice/timepoint/radiotracer), perform biodistribution by counting radioactivity in different tissue samples.

Specific Aim 4.4 Compare the biodistribution data obtained with radiolabeled exosomes with similar biodistribution data obtained using fluorescent labeled exosomes

Dr. Vallabhajosula's group tested the *in vivo* uptake of ^{124}I-exosomes (prepared using the Iodogen method described in Specific Aim 2 above) with microPET.

Experimental part:

1) Preparation of ^{124}I-labeled exosomes.

Exosomes isolated from MDA-231 cells (lung-tropic) and MCF-7 (low-metastatic BC cells used as controls) cells were used for these studies.

Labeling with Na^{124}I (supplied by MSKCC) proceeded as described in Specific Aim 2 using 120-130ug of exosomes. Labeling yield was 58-63%. Labeling mixtures were purified on a PD-10 gel filtration column using PBS as eluate. Fractions F7-F9 from each preparation were pooled together (320-350uCi in 1.5mL) and then used for animal injection (RCP>98%).

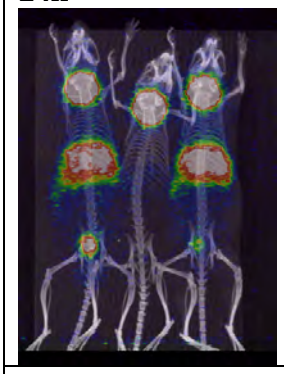
2) microPET imaging.

For PET studies, 6 female nude outbred mice (7 week old) were used. Two mice were injected with 60uCi ^{124}I-exos (20-25ug) from MDA-231 and two mice were injected with 60uCi ^{124}I-exos (20-25ug) from MCF-7 via the tail vein (administration volume: 0.25mL). As another control, the final two mice were injected with $Na^{124}I$ alone.
Following exosome administration, mice were imaged at 24h, 48h, 72h and 96h post administration using the Inveon PET/CT imaging system (Siemens). The imaging protocol was comprised of 5min CT for attenuation correction followed by 20min PET. Maximum intensity projection MIP images were generated using the Inveon Research Workplace (IRW) software.

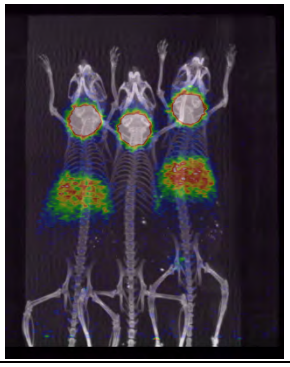
Results:

Figure 11 - MDA-231 (lung) exosomes

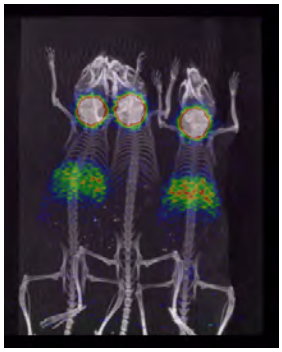
24h



48h



72h



96h

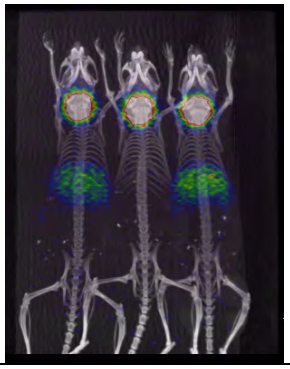
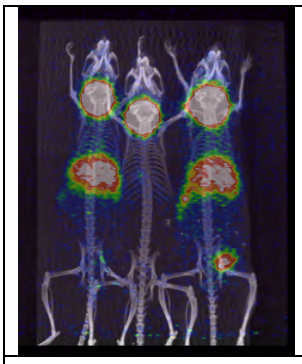
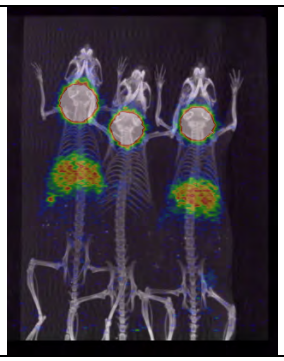


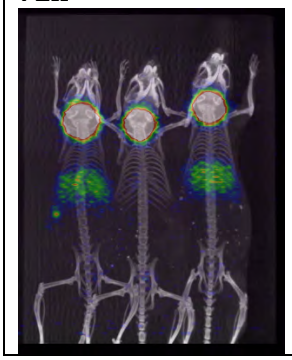
Figure 12 - MCF-7 exosomes

24h	**48h**

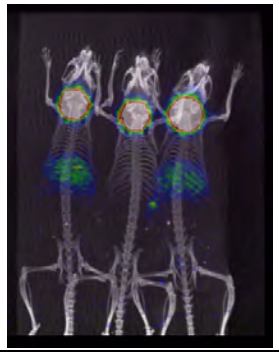




72h



96h



Whole body PET images of mice studied are presented in Figures 11 and 12. In both Figures the mouse in the middle of each image is the mouse injected with $Na^{124}I$ alone. That mouse shows high and exclusive thyroid uptake as expected due to the normal distribution of iodine to the thyroid gland.
Regarding the mice injected with radioiodinated exosomes, no difference in tissue distribution was observed between MDA-231 and MCF-7. High radioactivity uptake was evident in thyroid, liver and in some cases in bladder that was considered non-exosome specific. *In vivo* imaging data suggested possible breakdown of radiolabeled exosomes before or after *in vivo* administration. These findings were consistent with the idea that the Iodogen method used for the ^{124}I-labeling might destroy the exosomes. This hypothesis was confirmed by the "mock" labeling and then the analysis of labeled exosomes by electron microscopy as described in Specific Aim 2 part above.

Specific Aim 5. Following establishment of a successful radiolabeling protocol for exosomes derived from BC cell lines, a) develop protocols for the preparation of radiolabeled exosomes isolated from the circulation of BC patients, and b) study the biodistribution and tumor uptake of radiolabeled exosomes in BC xenografts.
Research staff from Dr. Vallabhajosula and Dr. Lyden will be working together on this task.
Specific Aim 5.1 Purify exosomes from human blood (BC patient) and characterize, size, number and protein concentration of exosomes
Specific Aim 5.2 Prepare radiolabeled exosomes (based on the best labeling method identified in specific aim 2)
Specific Aim 5.3 Perform in vitro cell binding studies with tumor cells, BM cells, fibroblasts and endothelial cells.

Specific Aim 5.4 Perform in vivo imaging studies and kinetics of distribution studies in BC high malignant (MDA-231) xenografts.
Specific Aim 5.5 Correlate the biodistribution data with tissue distribution data obtained with fluorescent labeled exosomes.

Due to high variability of yields and lack of reproducibility we have decided for now not to pursue the analysis of BC patient exosomes, and this has been reflected in our previous report. This change in scope was accepted by the granting agency following last year's progress report submission. Therefore, we will focus on performing experiments with exosomes isolated from *in vitro* organotropic models of human breast cancer.

KEY RESEARCH ACCOMPLISHMENTS:

- Our data demonstrate that tumor-secreted exosomes distribute to metastatic organs retaining the metastatic organotropism of the cell of origin, therefore tumor exosomes can be used to track specifically metastatic sites
- We found that tumor exosomes fuse specifically with different resident cells in the lung, liver and brain. Our data suggest that specific targeting of these interactions could be used to prevent exosome fusion and therefore could potentially reduce or block metastasis.
- We developed a protocol to label exosomes with radioiodide ^{131}I (for SPECT) or ^{124}I (for PET) using the Iodogen method. Despite successful labeling, we demonstrated that this method is not well-tolerated by exosomes leading to loss of their integrity/change of their nature.
- We developed an alternative protocol to label exosomes with radioiodide ^{131}I (for SPECT) or ^{124}I (for PET). This protocol is based on a milder radioiodination method that uses a Bolton-Hunter reagent to indirectly label protein molecules. We demonstrated that this protocol is very well-tolerated by exosomes.
- We developed a protocol to functionalize exosomes with the NOTA chelator and then label with the radiometal ^{68}Ga (for PET). We demonstrated that this protocol is very well-tolerated by exosomes.
- Overall, our data support that radiolabeled tumor exosomes could be used to predict metastatic sites, further investigation in pre-clinical models will verify this hypothesis and optimize the labeling and delivery approach.

Future plans:

i. To radiolabel NOTA-modified exosomes with Copper-64 (Cu-64). Cu-64 is a positron emitter with a half-life ($t_{1/2}$= 12h) higher than that of Ga-68 ($t_{1/2}$= 1h) and most probably more compatible with the pharmacokinetics of exosomes.
ii. To radiolabel exosomes indirectly using an anti-CD9 mAb. The anti-CD9 mAb will be first conjugated with a bifunctional chelating agent such as DOTA or DFO and then labeled with ^{111}In or ^{64}Cu (for DOTA) and ^{89}Zr (for DFO).

iii. To determine the stability of all radiolabeled exosome preparations: i) in storage and ii) in the presence of blood serum.
iv. Imaging studies with ^{131}I-BH-exosomes (MDA-231 and MCF-7) and ^{124}I-BH-exosomes (MDA-231 and MCF-7).
v. Tissue distribution studies with ^{131}I-BH-exosomes (MDA-231 and MCF-7) and ^{124}I-BH-exosomes (MDA-231 and MCF-7).
vi. Imaging studies with NOTA-modified exosomes (MDA-231 and MCF-7). The radiolabel will be selected based on the additional results of Specific Aim 2.
vii. Tissue distribution studies with NOTA-modified exosomes (MDA-231 and MCF-7). The radiolabel will be selected based on the additional results of Specific Aim 2.
viii. Imaging and tissue distribution studies of any additional radiolabeled exosome preparation that will be developed during Specific Aim 2.

REPORTABLE OUTCOMES: Provide a list of reportable outcomes that have resulted from this research to include:

Not applicable. No reportable outcomes are associated with this report.

CONCLUSION:

We have confirmed that BC exosomes can be efficiently radiolabeled with ^{124}I (for PET), ^{131}I (for SPECT) and ^{68}Ga (for PET) using two different labeling protocols that are well tolerated by exosomes. During Year 3, we will test the ability of these radiolabeled exosomes to be used as radioactive probes *in vivo*, for non-invasive molecular imaging studies using single photon emission computed tomography (SPECT) and positron emission tomography (PET) in order to predict metastatic spread and metastatic niches. During Year 3, we will also test some additional labeling protocols and evaluate the generated radiolabeled exosomes in cells and animals.

REFERENCES: Not applicable. No references are associated with this report.

APPENDICES: Not applicable. No appendices are attached to this report.

SUPPORTING DATA: Figures 1-12.